Extraction of functional ingredients from spinach (*Spinacia oleracea* L.) using liquid solvent and supercritical CO_2 extraction

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Keywords: Spinach leaves, extraction techniques, carotenoids, total polyphenol content, antioxidant activity, anti-inflammatory activity

Abstract

BACKGROUND: In this work three different techniques were applied to extract dry leaves of spinach (*Spinacia oleracea*): solid-liquid extraction (SLE), pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) to investigate the influence of extraction solvent and technique on extracts composition and antioxidant activity. Moreover, the influence of carotenoids and phenolic compounds on the antioxidant and anti-inflammatory activities of spinach extracts was also studied.

RESULTS: The higher concentrations of carotenoids and the lower content of phenolic compounds were observed in the supercritical CO_2 extracts; whereas water and/or ethanol PLE extracts presented low amounts of carotenoids and the higher concentrations of phenolic compounds. PLE extract with the highest content of phenolic compounds shows the highest antioxidant activities, although SFE carotenoid rich extract also shows a high antioxidant activity. Moreover, both extracts present an important anti-inflammatory activity.

CONCLUSION: PLE seems to be a good technique for the extraction of antioxidant and anti-inflammatory compounds from spinach leaves. Moreover, spinach phenolic compounds and carotenoids present a high antioxidant activity, whereas spinach carotenoids seem to show a higher anti-inflammatory activity than phenolic compounds. It is worth noting that of our knowledge this is the first time the anti-inflammatory activity of lipophilic extracts from spinach leaves is reported.

INTRODUCTION

In the last decades, the search of natural phytochemicals to be applied in foods, cosmetics, etc., has produced a growing interest in extraction and isolation techniques. Solid-liquid extraction is the most traditional technology used to extract active compounds from plant matrix. It is widely known that higher temperatures favor the solubility of the solute in the solvent and thus improve its recovery. Nevertheless, SLE temperature is limited by solvent boiling and in some cases due to the loss of volatile compounds. In this regard, pressurized liquid extraction allows the use of solvents in a liquid state at higher temperatures. Furthermore, a compression effect is made on the vegetal particle, which also contributes to improve extraction yield, lower amount of solvent is required, extraction is faster, higher yields are attained and the loss of volatiles is minimized.[4]

However, both SLE and PLE require a post-extraction procedure to separate the solvent from the extract, while supercritical fluid extraction using pure gases allows the recovery of the extract with high purity, completely free of solvent. The most employed solvent is CO_2 and selectivity is mainly determined by its density, which could be considerably varied by selecting adequate supercritical conditions (temperature and pressure). Carotenes are quite soluble in supercritical CO_2 and thus could be satisfactorily extracted by this technique without using polar cosolvents.[5] Yet, if ethanol is added as cosolvent the extraction of carotenoids from different vegetables is improved.[6] In this case, although the recovery of the extract can be performed in a depressurization stage without additional costs, further separation of the cosolvent from the product is unavoidable.

Spinach (*Spinacia oleracea*) is an edible flowering plant (Amaranthaceae family) native to central and southwestern of Asia and widely cultivated all over the world as one of

the most popular vegetables. It is identified as a good source of vitamin A, C, E, folic acid, minerals[7–9] as well as other bioactive compounds such as phenolics, carotenoids, glycoglycerol lipids[3] and lipoic acid.[10]

Several works are available in the literature, reporting the SLE of spinach leaves using water[2,11], methanol[12] and methanol:water mixtures[13]. Also, some studies have been focused on the extraction of spinach leaves with mixtures of acetone and water[2]. In these extracts, phenolic compounds such as flavonols and flavone glycosides derivatives, together with hydroxycinnamic acid derivatives were identified as the main phenolic compounds. [12,14] They are mainly reported to possess an important antioxidant activity[11,15] although other bioactivities such as anti-inflammatory,[16] antimutagenic and antiproliferative properties are also shown in biological systems.[17]

Furthermore, spinach has been suggested to be a vegetable that possess one of the highest amounts of lipophilic antioxidants such as carotenoids (mainly lutein, β-carotene and violaxanthin) and β and α-tocopherol.[9,18] Nevertheless, only few studies reported the antioxidant properties of organic spinach extracts.[19] Furthermore, β-carotene and lutein have been reported to possess anti-inflammatory activity[20–21] although, to the best of our knowledge, no reports about anti-inflammatory activities of lipophilic spinach extracts have been published.

PLE of fresh spinach was studied by Barriada-Pereira et al.[22] to determine the organochlorine pesticides present in the plant. Moreover, to our knowledge, only the work of Howard and Pandjaitan[23] reported the PLE of spinach with the target of extracting bioactive compounds. Similarly, not many studies have been conducted about the SFE of spinach, being the main target the recovery of diacylglycerols.[24]

In this work the SLE, PLE and SFE of dry spinach leaves were accomplished using different solvents (water, ethanol, ethanol: water mixture, hexane and pure supercritical

CO_2) with the target of investigate the influence of extraction solvent and technique on extracts composition and antioxidant activity. Moreover, the influence of carotenoids and phenolic compounds on the antioxidant and anti-inflammatory activities of spinach extracts was also studied. Temperatures explored were in the range 40-80°C; higher extraction temperatures were not investigated due to the possible thermal degradation of carotenoids.[1] Pressures were according to the extraction technology applied, from 0.1 MPa (SLE) to 35 MPa (SFE).

MATERIALS AND METHODS

Samples and reagents

Standards, chemicals and reagents: Lutein standard (≥95%) was purchased from Extrasynthese (Genay Cedex, France) and β-carotene standard (≥95%) from Sigma-Aldrich (Madrid, Spain). ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] and potassium persulfate were purchased from Sigma-Aldrich (Madrid, Spain). Methanol, hexane, diethyl ether, petroleum ether and methyl t-butyl ether were HPLC grade from LabScan (Gliwice, Poland), triethylamine was from Sigma-Aldrich (Madrid, Spain) and ethanol absolute was purchased from PANREAC (Barcelona, España). Sodium sulfate anhydrous pure was purchased from LabScan (Gliwice, Poland) and potassium hydroxide, Folin-Ciocalteu reagent, sodium carbonate and sea sand washed (thin grain) were from PANREAC (Barcelona, Spain).

Preparation of samples: the spinach (*Spinacea oleracea* L.) sample consisted of dry leaves (water content < 49.0 g water kg^{-1} of leaves) purchased from an herbalist's producer (Murcia, Spain). The sample was ground in a cooled mill and sieved to size between 200 and 500 μm.

Extraction methods

Solid-liquid extraction (SLE): experiments were carried out using 1 g of sample with 100 mL of hexane, ethanol or water at 50ºC in a Stuart Orbital S150 shaker apparatus for 24 h. After extraction, supernatant was filtered through cellulose filter and finally hexane and ethanol were removed by evaporation under vacuum at 35°C using a rotavapor, and the extracts were finally dried up to constant weight in a stream of N_2. Water extracts were freeze-dried. All experiments were carried out by duplicate. The dried samples obtained were stored at 4°C in the dark until analysis.

Pressurized solvent extraction (PLE): extractions were carried out in an ASE 350 system from Dionex Corporation (Sunnyvale, CA, USA) equipped with a solvent controller unit. Each extraction cell (10 ml capacity) was filled with 1 g of solid sample and 1 g of sea sand as a sandwich, and then placed into an oven. Then, the cell was filled with the corresponding solvent (hexane, ethanol, water or a mixture 50:50 ethanol: water) up to a pressure of 1500 psi and was heated-up to the desired temperature (80ºC). Static extractions were performed for 10 min. Preliminary studies (data not shown) revealed that higher extraction times had no significant effect on extraction yield. After extraction the cell was washed with the solvent and subsequently the solvent was purged from cell using N_2 gas until complete depressurization was accomplished. The extracts were recovered in glass vials and the solvent was eliminated as specified for solid-liquid extractions. All experiments were carried out by duplicate. The dried samples obtained were stored at 4 ºC in the dark until analysis.

Supercritical fluid extraction (SFE): trials were carried out in a pilot-plant scale supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell with automatic control of temperature and pressure. For each experiment, the cell was filled with 0.5 kg of plant raw material. The

extractions were performed at 40 and 70°C and two different pressures (25 and 35 MPa) were employed. The extraction time was 6 h and the supercritical solvent (CO_2) flow rate was set to 60 g min^{-1} in all experiments. The supercritical extract was separated in two fractions by means of a depressurization cascade system comprised by two separators (S1 and S2). Fractionation was accomplished by maintaining S1 at 10 MPa while S2 was set at the recirculation CO_2 pressure (5 MPa).

Ethanol was used to wash out the collector vessel and ensure a complete recovery of the material precipitated in the cell. Ethanol was eliminated by evaporation and the homogeneous solid samples obtained were kept at 4°C in the dark until analysis.

Chemical analysis

Determination of total carotenoid content: carotenoids were extracted from 40 mg of the dried extracts with 4 mL of methanol. Previous to analysis, samples were conducted to a saponification reaction in order to remove the chlorophylls that could interfere in the spectrophotometric determination. For saponification the method proposed by Granado et al.[26] was followed, but the mixture of petroleum ether plus diethyl ether (50:50) was replaced by diethyl ether (100%). The saponified extracts were dissolved in petroleum ether (1 mg mL^{-1}) and the carotenoid content measured spectrophotometrically at 450 nm. Quantification was performed by using an external standard of pure β-carotene and the results were expressed as equivalents of mg β-carotene g^{-1} extract.

Identification and quantification of lutein and β-carotene: the analysis of carotenoids was based in the method proposed by Breithaupt[25] but using a C18 column instead of a C30 column. Samples obtained after saponification were analyzed employing a HPLC model Agilent 1260 Infinity (Agilent, Santa Clara, USA) equipped with a KROMASIL

100 C18 column (Scharlab, Barcelona, Spain) of 25 mm × 4.6 mm and 5 μm particle size. The mobile phase comprises solvent A, which is a mixture of methanol:water (90:10) and triethylamine 1 mL L^{-1} and solvent B, containing methyl-tert-butyl ether:methanol:water (90:6:4) and triethylamine 1 mL L^{-1}. The gradient started with 93 % A to 0 % A from 0 to 34 min and recovers the initial conditions of the method in 4 min. Total time analysis was 38 minutes. During analysis the column was maintained at 25ºC. The flow rate was constant at 1 mL min^{-1} and the injection volume was 20 μL. For detection were assigned the wavelength of 450, 470, 550, 660 nm. For quantification of carotenoids calibration curves were performed with commercial standards of β-carotene and lutein, from which straight lines were obtained with a linear regressions of R^2=0.9984 and R^2=0.9996, respectively.

Determination of total polyphenol content (TPC): total phenolic content was determined using the colorimetric method developed by Singleton et al.[27] Results were expressed as gallic acid equivalents (GAE) (mg of gallic acid g^{-1} extract) using a standard curve of gallic acid. Triplicate measurements were carried out.

Determination of antioxidant activity

$ABTS^{\bullet+}$ assay. The $ABTS^{\bullet+}$ assay described by Re et al.[28] was used to measure the antioxidant activity of the spinach extracts. The reaction was carried out at four different concentrations of extract and was allowed to stand until the absorbance reached a plateau, and the absorbance was recorded at 734 nm. Trolox was used as reference standard, and results were expressed as TEAC values (mmol TE g^{-1} extract). All analyses were done in triplicate.

Determination of anti-inflammatory activity

Cell culture and treatment: Human THP-1 monocytes (American Type Culture Collection, ATCC) were cultured in RPMI 1640 culture medium (Gibco, Spain) supplemented with 100 mg kg^{-1} FBS, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 2 mmol L^{-1}-glutamine and 0.005 mmol L^{-1} β-mercaptoethanol at 37ºC in 95% humidified air containing 5% CO_2. Cells were collected and plated at a density of $5x10^5$ cells mL^{-1} in 24 wells plates. Differentiation to macrophages (THP-1/M cells) was induced by maintaining the THP-1 cells in the presence of 100 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA) (Sigma, Spain) for 48h. After differentiation, cells were washed with PBS and incubated with 0.05 µg mL^{-1} LPS in presence of different concentrations of spinach extracts for 24h in a FBS free medium. Then, the supernatant was frozen at -80ºC.

Quantification of cytokines by ELISA: The release of IL-1β, IL-6 and TNF-α was measured in the supernatants of THP-1/M cells treated with LPS in presence of different concentrations of spinach extracts using ELISA kits (BD biosciences, Spain), according to manufacturer's instructions. The color generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise, Tecan).

Statistical analysis

Experimental results are expressed as means ± standard deviation (SDs). One-way analysis of variance (ANOVA) was used to look for differences between means at a 95.0% confidence level. Multiple range test was used to distinguish which means were significantly different from which others. Statistical analyses were performed using Statgraphics v. Centurion XVI for Windows (Statistical Graphics, Washington, USA)

RESULTS AND DISCUSSION

Extraction yield and content of carotenoids

Tables 1 to 3 shows the extraction yields obtained in the SLE, PLE and SFE of spinach leaves. The data reported correspond to the mean values (MV) obtained between duplicates. Standard deviations (SD) obtained in the SLE and PLE extraction yields are also given in the tables. Relative deviations (SD/MV) were lower than 7% in SLE and 20% in PLE assays. The mean relative deviations obtained in the S1 and S2 fractions collected in the SFE experiments was 8%.

In general, extraction yields obtained when using liquid polar solvents, such as water, ethanol or ethanol: water mixture, are considerably higher (one order of magnitude higher) than yields obtained using non-polar solvents, such as liquid hexane and supercritical CO_2.

In comparison with liquid extraction at ambient pressure and 50°C, PLE produced a 1.5 fold increase in the case of hexane. This important increase of yield could be attributed to the PLE temperature (80°C) which is higher than the normal boiling point of the solvent employed. This significant increase of the solvent power, that is produced when the extraction temperature became higher than its normal boiling point, was previously observed and reported.[29] On the contrary, the highest extraction yield using water was obtained in SLE and not in PLE. This lower yield observed in water PLE could be attributed to an extraction temperature lower than water normal boiling point, and the considerable shorter extraction times applied (10 min vs. 24 h).

Iijima et al.[24] reported yields of 14.4 mg g^{-1} and 351.7 mg g^{-1} for the ultrasound assisted SLE of freeze-dried spinach using, respectively, hexane and methanol. These values are of the same order of magnitude of the yields obtained in this work (28.4 mg g^{-1} with hexane, 101.4 mg g^{-1} with ethanol and 305.8 mg g^{-1} using water) taking into account the polarity of the solvents employed. No comparison can be established with respect to

PLE yields, since no overall extraction yields were reported in previous spinach PLE studies.[22–23]

Extraction yields in the SFE assays were the lower ones. Lower extraction temperature (40°C) produced higher extraction yields (S1+S2), despite the extraction pressure applied. This tendency was also reported by Iijima et al.[24] although the values obtained were slightly larger than the yields obtained in this work. For example, at 25 MPa extraction yields reported are 35.1 and 29.1 mg g^{-1} at 40 and 70°C, while extraction yields obtained in this work are, respectively, 21.6 and 19.4 mg g^{-1}. Yet, it should be taken into account that, maintaining the same extraction conditions, differences in SFE yields up to 400 mg g^{-1} were found for different *Spinacia oleracea* subspecies.[24] With respect to the on-line fractionation of the supercritical extract, yields obtained in S2 were considerably higher than those obtained in S1 at 40°C, while similar yields were obtained in S1 and S2 at 70°C.

As mentioned before, the main carotenoids identified in the samples were lutein and β-carotene. Tables 1 to 3 show the content of these carotenoids as determined by the HPLC analysis. Hexane SLE, ethanolic PLE extract and supercritical CO2 extracts produced the samples with the higher concentration of these carotenoids. Lutein recovery (mg carotenoid / g dry matter) was significantly higher in the case of the ethanolic PLE extract (0.49 mg/g) in comparison with the hexane SLE extract (0.04 mg/g) or the supercritical CO_2 extracts (0.03-0.06 mg/g). On the contrary, β-carotene recovery was higher in the case of hexane and CO_2 extraction (0.10-0.38 mg/g) in comparison with the ethanolic PLE extract (0.07 mg/g). These differences can be attributed to the higher polarity of xanthophylls in comparison with carotenes, due to the presence of hydroxyl groups in their chemical structure.

In general, the concentration of β-carotene in these samples is higher than that of lutein (3-5 times higher in S1+S2 supercritical extracts and almost 10 times higher in solid-liquid extraction with hexane). SFE yields were higher at 40°C, however carotenoid concentrations were higher in the assays carried out at 70°C. For example, at 35 MPa, the total content (S1+S2) of lutein and β-carotene were, respectively, 1.6 and 4.9 mg g^{-1}at 40°C, and 3.5 and 13.7 mg g^{-1} at 70°C. Furthermore, the cascade depressurization system in SFE resulted in partial fractionation of these carotenoids: in the extraction accomplished at 40°C, the concentration of lutein in S1 samples were around 2 times higher than that of β-carotene, while the concentration of β-carotene in S2 samples were 10 times higher than that of lutein. Table 3c also show the total content of carotenoids determined in the SFE extracts. Values varied from 16 to 32 mg g^{-1}, representing lutein and β-carotene around 50% of the total amount of carotenoids identified. Furthermore, increased amounts of total carotenoids were obtained in S1 samples (from 33 to 65 mg g^{-1}) than in S2 samples (from 12 to 16 mg g^{-1}).

Bunea et al.[9] studied the carotenoid content of fresh, refrigerated and processed spinach (*Spinacia oleracea*). According to their work, fresh spinach contains 18-31 mg β-carotene kg^{-1} of fresh matter, what represents around 0.2-0.4 mg β-carotene g^{-1} of dried matter (water content in spinach was approximated to be 920 g kg^{-1}). These values are in accordance with those obtained in our work, i.e. the extraction of 0.21 mg of β-carotene g^{-1} dried matter by SFE (at 35 MPa and 70°C, see Table 3) and 0.38 mg of β-carotene g^{-1} dried matter by SLE with hexane (see Table 1).

Antioxidant activity of the extracts

PLE (Table 2) seems to be a good technique to extract antioxidant compounds from spinach leaves, as higher TEAC values were obtained in PLE extracts compared with

conventional solvent extraction (Table 1) and SFE extraction (Table 3). Both PLE and SLE showed the same behavior of the extracts regarding the solvent used. Water extracts possessed the highest antioxidant activity, closely to ethanol extracts. The lowest TEAC values were obtained with hexane. These results are in accordance with Pellegrini et al.[19], where better results were achieved using water than chloroform in ultrasound assisted SLE, although slightly higher activities in the extracts were reported, probably due to the use of different cultivars or growing season.[15] Many other studies have shown the antioxidant activity of spinach extracts, however as other methods different to ABTS assay were used no direct comparisons with our results were able to establish.[23,30]

Regarding to SFE, and in all extraction conditions explored, the samples obtained in S1 presented higher TEAC values than those recovered in S2 (Table 3d). This effect could be attributed to the higher content of carotenoids determined in S1 samples (Table 3c) as is explained in the following section. Furthermore, TEAC values of S1 SFE extracts were intermediate between PLE and conventional solvent extraction, and in general closely related to the PLE hexane extraction. Furthermore, greater effect of SFE extraction conditions was found on the antioxidant capacity of S1 samples in comparison of S2 samples: better results in S1 extracts were produced when lower extraction temperature were applied (40ºC vs. 70ºC), whereas only a slight effect on antioxidant capacity were shown increasing extraction pressure.

Aqueous and ethanolic extracts from PLE showed the highest contents of phenolic compounds, while hexane or SFE extracts possessed considerably lower concentrations. It has been reported that lipophilic substances such as tocopherols or phospholipids can react with Folin-Ciocalteu reagent causing an overestimation of the TPC. In this way, TPC of hexane or SFE extracts could be attributed to interferences with other

substances rather than the presence of phenolic compounds in the extracts.[31] Moreover, no flavonoids or hydroxycinnamic acids are expected to be extracted with non-polar solvents such as hexane or supercritical CO_2.[19]

Bunea et al.[9] also determined the content of total phenolic compounds in spinach using the Folin-Ciocalteu reagent and gallic acid as standard, reporting that fresh spinach contains around 27 mg GAE g^{-1} of dried matter (again, water content in spinach was estimated to be 920 g kg^{-1}). According to our work, the maximum amount of phenolic compounds was extracted from spinach by PLE with ethanol, attaining 18.4 mg g^{-1} dried matter (see Table 1).

Pellegrini et al[19] published an interesting study about the efficiency of extraction of a sequence of solvents on spinach leaves. They found acetone as the best solvent for the extraction of carotenoids followed by chloroform, while water caused no extraction of carotenoids. On the other hand, water turned out a high extraction of phenolic compounds followed by acetone. No polyphenols were found in chloroform extract. Similar results were obtained in this study, since better polyphenolic contents were found in water extracts followed by ethanol, while carotenoids were better extracted with ethanol than hexane in PLE and conventional solvent extraction.

As different polyphenol and carotenoid content were found in the extract regarding to solvent, it seems that TEAC values of PLE and conventional solvent extraction with water is related to the presence of phenolic compounds, opposite to hexane extracts or SFE extracts where antioxidant activity could be due to the presence of carotenoid compounds. Ethanolic extracts antioxidant activity could be related to both, phenolic and carotenoids compounds.

Figure 1 shows the TEAC values obtained for all samples produced as a function of the content of TPC (Figure 1a) and carotenoids (Figure 1b). Despite the antioxidant activity

may well be attributed to the presence of carotenoids, phenolic compounds or both type of substances, is clearly deduced from Figure 1 that the content of phenolic compounds has the dominant effect on the antioxidant capacity of spinach extracts, except for the SFE extracts. Many studies have reported a linear relationship between TPC and antioxidant activity in aqueous, ethanolic or methanolic extracts.[15,32] In this regard, although no linear relationship was found between TPC and TEAC values, the highest antioxidant activity corresponds to the extracts with the higher content of TPC, namely the PLE water:ethanol (50:50) spinach extract is the one with the higher TEAC value (0.369 mmol TE g^{-1}) and higher content of phenolic compounds (88.839 mg GAE g^{-1}). Moreover, carotenoids may exert a clear influence in TEAC value of the SFE extracts with no influence of TPC. Furthermore, while no correlation between the content of β-carotene + lutein and TEAC values can be established in the case of PLE extracts, it is clearly observed in Figure 1b that the content of carotenoids have a great influence on the TEAC values of SFE extracts.

Anti-inflammatory activity of the extracts.

The anti-inflammatory capacity of the PLE water:ethanol (50:50) spinach extract (80°C) and the SFE S1 extract (40°C and 35 MPa) was measured using THP-1 human macrophages activated with LPS. These extracts were specifically chosen because the PLE extract presented the highest concentration of phenolic compounds and the highest antioxidant activity, while the SFE extract presented the highest content of total carotenoids together with the highest TEAC value of the SFE extracts. In this regard, it is pretended to asses if some particular type of compounds (phenolic compounds or carotenoids) has larger effect on the anti-inflammatory activity of spinach extracts.

The activation of THP-1/M was carried out with the addition of LPS to the medium. These LPS treated cells showed, after an incubation period of 24h, an important increase in the release of all anti-inflammatory cytokines tested (TNF-α, IL-1β and IL-6) compared to non-activated controls (Figure 2). These activated cells were considered as positive controls for all the cytokines tested. When the activation of THP-1/M was carried out in presence of 20 μg mL^{-1} of spinach extracts, a small decrease in TNF-α secreted level was observed when used SFE extract (Figure 2), compared with levels obtained in absence of extracts (positive control). However, no significant decrease in the amount of TNF-α secreted was obtained with 20μg mL^{-1} PLE extract. Regarding to IL-1β secretion by activated cells in presence of spinach extracts (Figure 2), it can be observed an important decrease in the secretion of this cytokine. Thus, 20μg mL^{-1} of the SFE extract reduced a 50% the release of IL-1β, meanwhile PLE extract only presented a 30% of inhibition, compared to positive control. The activation of macrophages in presence of extracts also produced an important decrease in the IL-6 release (Figure 2), overall with SFE extracts which inhibit an 80% the IL-6 secretion. These data indicated that supercritical spinach extract presented an important anti-inflammatory activity in THP-1 human macrophages activated with LPS, since only 20 μg mL^{-1} of this extract effectively inhibited the release of pro-inflammatory cytokines. Therefore, a higher anti-inflammatory activity was shown in the SFE extract than in the PLE extract. SFE extract activity could be attributed to the important quantity of lutein and β-carotene detected in the SFE extract, since several studies have reported the anti-inflammatory effects of lutein or β-carotene.[33–34] On the other hand, considering that neither lutein nor β-carotene were identified in the PLE extract, its anti-inflammatory activity could be related to the presence of phenolic compounds since several authors have reported the anti-inflammatory effect of these compounds.[35] In this regard, carotenoids may play an

important role in the anti-inflammatory activity of the lipophilic extracts. Moreover, although more studies should be done it seems that carotenoid-rich extracts would show a higher anti-inflammatory effect than polyphenol-rich extracts from spinach leaves.

CONCLUSIONS

PLE seems to be a good technique for the extraction of antioxidant compounds from spinach leaves, although good results were also achieved in some SFE extracts. In this regard, solvent polarity makes conditional the composition of the extracts; that is, pressurized water or ethanol:water at 80ºC produced the highest polyphenols concentration whereas the highest carotenoids concentration was achieved using supercritical CO_2. Both extracts show a high antioxidant activity being attributed to the polyphenol or carotenoid content, respectively. Moreover, both extracts show anti-inflammatory activity too, although higher activity was found in SFE extract. Therefore, it is demonstrated that spinach phenolic compounds and carotenoids present a high antioxidant activity, whereas spinach carotenoids seem to show a higher anti-inflammatory activity than phenolic compounds. Furthermore, it is worth noting that of our knowledge this is the first time the anti-inflammatory activity of lipophilic extracts from spinach leaves is reported.

Acknowledgements

This work has been supported by project INNSAMED IPT-300000-2010-34 (subprogram INNPACTO) from Ministry of Science and Innovation (Spain) and project ALIBIRD-S2009/AGR-1469 from Autonomous Community of Madrid.

The authors Laura Jaime, Tiziana Fornari, Mónica R. García-Risco, Susana Santoyo and Guillermo Reglero are members of the IBERCAROT network, funded by CYTED (ref. 112RT0445).

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Table 1. SLE of spinach leaves: extraction yield (g mass extract kg^{-1} mass vegetal matrix), content of lutein and β-carotene (mg carotenoid g^{-1} extract) and antioxidant activity (mmol trolox g^{-1} extract) obtained in the extracts. Extraction temperature = 50°C.

Solvent	Extraction yield (g kg^{-1})	Content of main carotenoids identified (mg g^{-1})		TEAC value (mmol TE g^{-1})	TPC (mg GAE g^{-1})
		Lutein	β-carotene		
Water	$308.0 \pm 22.0^{a*}$	ND.	ND	$0.198 \pm 0.012^{a*}$	$25.256 \pm 1.354^{a*}$
Ethanol	101.4 ± 1.2^{b}	2.41	3.05	0.170 ± 0.005^{b}	23.851 ± 0.468^{b}
Hexane	28.4 ± 1.7^{c}	1.40	13.33	0.116 ± 0.002^{c}	5.135 ± 0.043^{c}

ND: not detected

*Different superscript letters within a column denotes statistically significant differences ($P \leq 0.05$) among solvents.

Table 2. PLE of spinach leaves: extraction yield (g mass extract kg^{-1} mass vegetal matrix x 100), content of lutein and β-carotene (mg carotenoid g^{-1} extract), antioxidant activity (mmol trolox g^{-1} extract) and total polyphenols content (mg gallic acid g^{-1} extract) obtained in the extracts. Extraction temperature = 80°C.

Solvent	Extraction yield (g kg^{-1})	Content of main carotenoids identified (mg g^{-1})		TEAC value (mmol TE g^{-1})	TPC (mg GAE g^{-1})
		Lutein	β-carotene		
Water	$189.9 \pm 2.7^{a*}$	ND.	ND	$0.329 \pm 0.013^{b*}$	$68.542 \pm 11.289^{b*}$
Ethanol	93.2 ± 8.5^{bc}	5.27	0.81	0.314 ± 0.011^{b}	58.236 ± 3.287^{c}
Ethanol:water (50:50)	104.2 ± 13.1^{b}	0.12	n.i.	0.369 ± 0.013^{a}	88.839 ± 2.345^{a}
Hexane	41.1 ± 8.2^{d}	1.99	0.23	0.186 ± 0.018^{c}	12.502 ± 3.133^{d}

ND: not detected

*Different superscript letters within a column denotes statistically significant differences ($P \leq 0.05$) among solvents.

Table 3. SFE of spinach leaves.

(a) Extraction yield (g mass extract kg^{-1} mass vegetal matrix)

T (°C)	P (MPa)	Extraction yield in separators		Overall extraction yield (S1 + S2)
		S1	S2	
40	25	1.0	20.6	21.6
40	35	1.2	19.7	20.9
70	25	6.3	8.1	14.4
70	35	10.6	5.3	15.9

(b) Content of lutein and β-carotene (mg carotenoid g^{-1} extract)

T (°C)	P (MPa)	lutein			β-carotene			lutein + β-carotene in S1+S2 extracts
		S1	S2	S1+S2	S1	S2	S1+S2	
40	25	17.29	0.64	1.41	10.39	6.61	6.80	8.21
40	35	20.80	0.43	1.60	8.02	4.80	4.94	6.54
70	25	5.81	0.37	2.75	10.42	5.79	7.85	10.59
70	35	4.87	0.87	3.53	17.01	7.10	13.67	17.21

(c) Total carotenoid content (mg β-carotene g^{-1} extract)

T (°C)	P (MPa)	Total carotenoids		
		S1	S2	S1+S2
40	25	42.9	15.8	17.1
40	35	65.2	13.1	16.0
70	25	32.6	14.5	22.4
70	35	41.6	11.9	31.7

(d) Antioxidant activity and total polyphenols content obtained in the extracts.

T (°C)	P (MPa)	TEAC value (mmol TE g^{-1} extract)		TPC (mg GAE g^{-1} extract)	
		S1	S2	S1	S2
40	25	$0.190 \pm 0.008^{b*}$	$0.080 \pm 0.004^{c*}$	$39.17 \pm 3.23^{a*}$	$7.86 \pm 0.55^{d*}$
40	35	0.275 ± 0.012^{a}	0.080 ± 0.001^{c}	19.84 ± 0.42^{d}	13.91 ± 0.54^{c}
70	25	0.132 ± 0.005^{d}	0.086 ± 0.003^{b}	27.12 ± 2.50^{b}	17.35 ± 1.45^{a}
70	35	0.153 ± 0.004^{c}	0.104 ± 0.004^{a}	24.79 ± 0.67^{c}	14.82 ± 0.27^{b}

*Different superscript letters within a column denotes statistically significant differences ($P \leq 0.05$) among solvents.

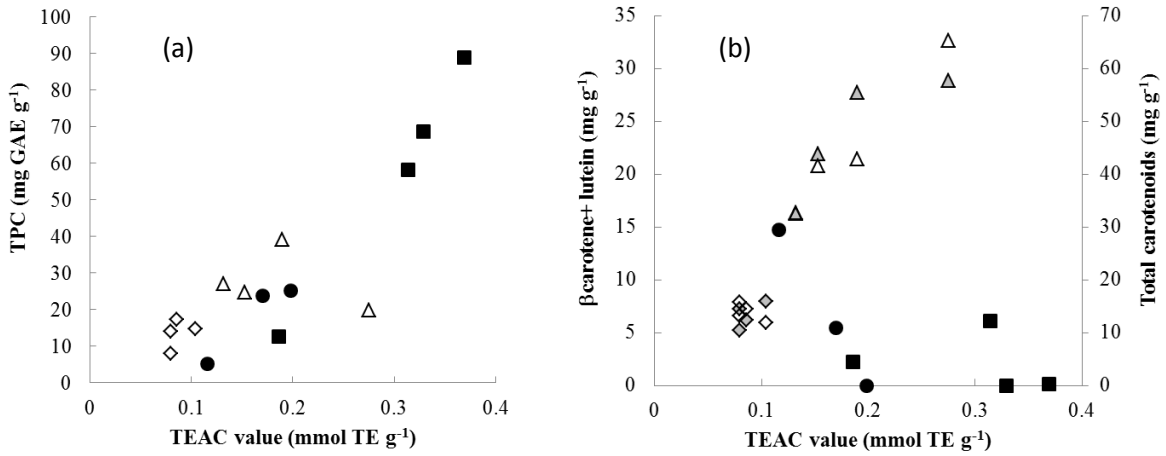

Figure 1. Correlation of TEAC (mmol TE g^{-1}) of spinach extracts with (a) total phenolic compounds (TPC) content, (b) carotenoid content: (■) PLE; (●) SLE; (△), (◈) β-carotene + lutein content in SFE-S1 and SFE-S2 extracts; (△), (◇) total carotenoids in SFE-S1 and SFE-S2 extracts.

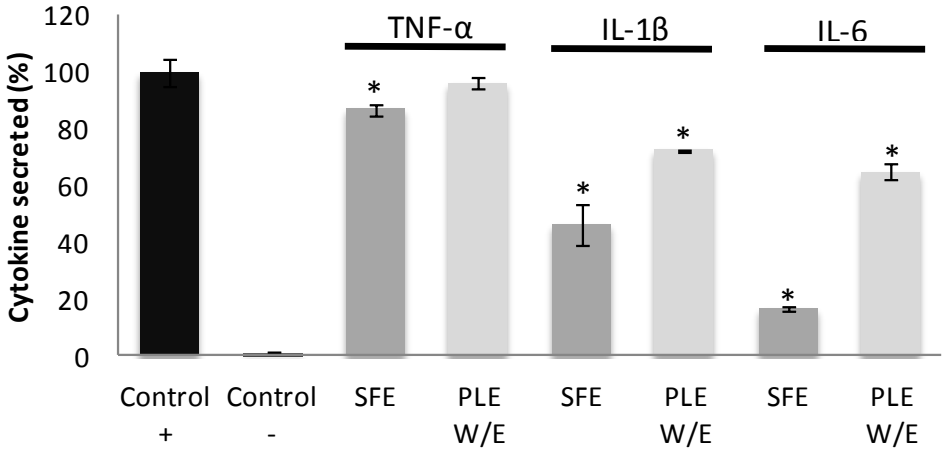

Figure 2: Levels of TNF-α, IL-1β and IL-6 secreted by THP-1/M activated with LPS in presence of spinach extracts: SFE (supercritical extract) and PLE W/E (extract obtained by pressurized liquids water:ethanol 1:1). Each bar is the mean of three determinations ± standard deviation.